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Phylogenetic Analysis of the Caliciviruses

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A phylogenetic portrait of the genus *Calicivirus* in the family *Caliciviridae* was developed based upon published sequences and newly characterized calicivirus (CV) strains, including additional Sapporo-like HuCV strains in pediatric diarrhea stool specimens from South Africa, the United Kingdom, and the United States. Distance and parsimony methods were applied to nucleotide and amino acid sequences of human and animal calicivirus 3D RNA-dependent RNA polymerase (~470nt) and capsid hypervariable regions (~1,200nt) to generate phylogenetic trees. Pairwise amino acid identity in the 3D region among the Sapporo-like strains ranged from 61% to 100%. Human and animal caliciviruses (HuCVs and AnCVs) separated into five genogroups: small round-structured viruses (SRSV), Sapporo-like, and hepatitis E virus (HEV)-like HuCVs and rabbit-, and vesicular exanthema of swine virus (VESV)-like AnCVs, each with a distinct genome organization. Each genogroup, including the Sapporo-like HuCVs, subdivided further into subgenogroups. The capsid region trees had higher levels of confidence than the 3D region trees and limited conclusions about genogroups could be drawn from the 3D region analyses. This analysis suggested that CVs include five potential virus subfamilies. ***J. Med. Virol. 52:419–424, 1997.***

KEY WORDS: calicivirus; genome; RT-PCR; phylogeny; distribution; diarrhea

INTRODUCTION

Caliciviridae were recognized to be taxonomically distinct from picornaviruses in 1978 [Cooper et al., 1978], based upon differences in particle morphology and the observation that CVs contain only one major structural polypeptide. Human strains cause sporadic cases and outbreaks of gastroenteritis [Matson et al., 1989]. In addition, hepatitis E virus is an enterically transmitted agent currently assigned to the *Caliciviridae* [Bradley and Balayan, 1988; Cubitt et al., 1995]. In animals, a variety of syndromes are caused by CVs, including myositis, encephalitis, mucosal ulcers, spontaneous abortion, and gastroenteritis [Barlough et al., 1986; Fastier, 1957; Smith and Boyt, 1990; Smith et al., 1973].

Caliciviruses are positive-sense, single-stranded, nonenveloped RNA viruses with a single capsid protein. Typical CVs have a distinctive appearance when visualized by direct electron microscopy (EM), presenting "calices" on the surface [Appleton, 1987], and include AnCVs such as San Miguel sea lion viruses (SMSVs) [Smith et al., 1973], rabbit hemorrhagic disease virus (RHDV) [Meyers et al., 1991], and feline CVs [Neill et al., 1991], and HuCVs associated with gastroenteritis, predominantly in children [Dinulos and Matson, 1994]. Sequencing results from a group of small, round structured, human enteric viruses (SRSVs), the best known of which is the Norwalk virus, have demonstrated that many of these also are CVs, despite a lack of the typical EM appearance [Jiang et al., 1993].

Genomic characterization of known and candidate CVs has led to recognition of considerable diversity within the family [Smith et al., 1973]. This report de-

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TABLE I. Sequences Included in Phylogenetic Analysis*

Source	Cryptogram or common name	Genbank number	Abbreviation
Human	HuCV/Houston/27/90/US	[U67859]	HuCV-27
	HuCV/London/29845/92/UK	[U67858]	HuCV-Lon
	HuCV/Vanderbijlpark/313616/93/SA	[U67857]	HuCV-Van
	HuCV/Sapporo/82/Japan	[S77903]	HuCV-Sap
	HuCV/Houston/DCC/86/US	[U67856]	HuCV-DCC
	HuCV/Plymouth/92/UK	[X86560]	HuCV-Ply
	HuCV/Manchester/93/UK	[X86559]	HuCV-Man
	HuCV/NV/8FIIa/76/US	[M87661]	HuCV-NV
	HuCV/KY/89/Japan	[L23828]	HuCV-KY
	HuCV/Southampton/91/UK	[L07418]	HuCV-Sou
	HuCV/SMA/76/US	[L23831]	HuCV-SMA
	HuCV/MX/89/Mexico	[U22498]	HuCV-MX
	HuCV/TV/91/Canada	[U02030]	HuCV-TV
	HuCV/Oth-25/89/Japan	[L23830]	HuCV-Oth
	HuCV/DSV/90/Saudi Arabia	[U04538]	HuCV-DS
	HuCV/Hawaii/71/US	[U07611]	HuCV-Haw
	HuCV/Bristol/93/UK	[X76716]	HuCV-Bri
	HEV/Burma-9/Burma	[M73218]	HEV-Burma
Animal	Rabbit hemorrhagic disease virus	[M67473]	RHDV
	European brown hare syndrome virus	[U09199]	EBHSV
	Feline calicivirus-CFI	[U13992]	FCV-CFI
	Feline calicivirus-F4	[D90357]	FCV-F4
	Feline calicivirus-F9	[Z11536]	FCV-F9
	Cetacean calicivirus Tur-1	[U52091]	CCV
	Reptile calicivirus Cro-1	[U52092]	RCV
	San Miguel sea lion virus-1	[M87481]	SMSV-1
	San Miguel sea lion virus-4	[M87482]	SMSV-4
	San Miguel sea lion virus-13	[U52087]	SMSV-13
	San Miguel sea lion virus-15	[U52088]	SMSV-15
	San Miguel sea lion virus-17	[U52094] & [U52005]	SMSV-17
	Primate calicivirus Pan-1	[U52086]	Pan-1

*Sequence from a picornavirus; human rhinovirus 14 [K02121], also was included.

scribes phylogenetic relationships underlying this genomic variation and expands knowledge of genomic diversity among Sapporo-like HuCVs by characterization of additional strains [Liu et al., 1995; Matson et al., 1995].

MATERIALS AND METHODS

Virus Sources

The source of strains newly characterized in this publication is provided. HuCV-27 was identified in rotavirus-negative diarrhea stool samples collected between November 1989, and December 1991, from children attending day care centers in Houston, Texas (Table I) [O'Ryan et al., 1994]. A diarrhea stool sample obtained from an 8-month-old infant in London, U.K., in October, 1992, who was hospitalized for diarrhea and vomiting, contained typical CV particles by EM (HuCV-Lon). A diarrhea stool sample collected in October 1993, from a 4-month-old boy in Vanderbijlpark, South Africa, south of Johannesburg, contained typical CV particles (HuCV-Van) [Wolfaardt et al., submitted]. Typical CV was identified by EM in sporadic cases of acute gastroenteritis in a 16-month-old girl in Plymouth (December 1992) and a 6-month-old boy in Manchester, U.K. (November 1993) (HuCV-Ply and HuCV-Man, respectively) [Liu et al., 1995].

Reverse Transcriptase-Polymerase Chain Reaction

Viral nucleic acid was extracted from 300–400 μl of 10–50% stool suspensions [Jiang and Matson, 1996]. The reverse transcriptase reaction was carried out on 1–5 μl of extracted nucleic acid using AMV reverse transcriptase (Promega, Madison, WI) and 2 μl of 0.1 μg/μl of first primer in a reaction volume of 40 μl. Polymerase chain reaction was carried out using *Taq* polymerase and 2 μl of 0.3 μg/μl of second primer in a reaction mixture of 88 μl [Jiang et al., 1992]. The parameters of the PCR were: 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 49°C, and elongation for 1 min 20 sec at 72°C.

Primers

Primer sequences were derived from the predicted RNA-dependent-RNA-polymerase (3D) region of available CV genomic sequences and purchased (Midland Certified Reagent Co., Midland, TX). Primers included NV35 [Jiang et al., 1992], Sapp36 [Matson et al., 1995], and Sapp35; 5′-GCA GTG GGT TTG AGA CCA AAG-3′.

Cloning and Sequencing of RT-PCR Products

The RT-PCR products were cloned into pGEM-T (Promega), and inserts were sequenced using ^{35}S-dideoxy nucleotide chain termination, "forward" and

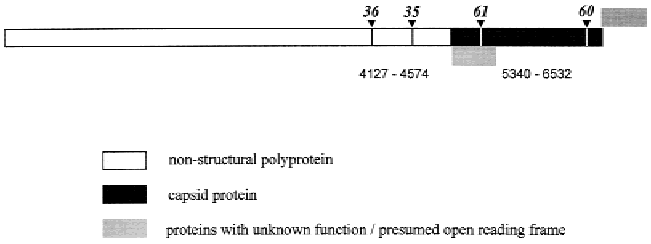

Fig. 1. Schematic diagram of the Sapporo-like HuCV genome organization. The location of the primer pairs 35/36 and 60/61 are indicated. Boxes represent open reading frames. Shadings indicate different confirmed or predicted genes [Liu et al., 1995].

"reverse" primers, and Sequenase (UBS Corp., Cleveland, OH). Radionucleotide-labeled products were resolved in 8% polyacrylamide gels and exposed to X-AR-2 film (Kodak, Rochester, NY).

Sequence Analyses

Nucleic acid sequences were manipulated and pairwise sequence analyses were performed using PC/Gene 6.85 (IntelliGenetics, Mountain View, CA). Multiple alignments of nucleic acid and predicted amino acid sequences were performed using CLUSTAL V [Higgins et al., 1992]. For the multiple alignment, homologous sequences were selected from the chosen genomes using the consensus amino acid motifs (YGDD [KGDD in HEV-Burma] motif in the 35–36 region and the PPG [PPN in MX-like] motif in the 60–61 region) shared among calici- and picornaviruses as anchors. The alignment of the motifs in the multiple alignment was verified by eye.

Analyzed Sequences

The sequences included in the phylogenetic analyses are listed in Table I. These include animal and human CVs from all hypothesized genogroups, based upon prior publications and preliminary analyses. These strains are all in the genus *Calicivirus* of the family *Caliciviridae* [Cubitt et al., 1995]. The inclusion of a strain's genomic region in a phylogenetic analysis depended upon availability of the given sequence (e.g., EBHSV, SMSV-1).

Phylogenetic Trees

Trees were constructed for the 3D region (~470 nucleotides) and a ~1.2Kb region (60/61) of the capsid gene containing the most hypervariable region of the capsid gene, with the aid of PHYLIP 3.5c [Felsenstein, 1993]. These regions correspond to the HuCV-Man virus sequence at 4127–4574 nt and 5340–6532 nt, respectively (Fig. 1).

Parsimony and distance methods were used for the phylogenetic tree constructions. Trees for both nucleotide and predicted amino acid sequences were established utilizing the programs PROTPARS and DNAPARS for the parsimony algorithms and PROTDIST and DNADIST for the distance matrices. The PAM and Kimura's distance estimates were used in the FITCH program, utilizing the Fitch-Margoliash method to build final distance trees [Felsenstein, 1993].

Statistical Analyses

The confidence values of the internal lineages within the dendrograms were assessed by a bootstrapped analysis [Felsenstein, 1985]. In each analysis, 1,000 bootstrapped data sets were created from which trees were constructed. A consensus tree of the bootstrapped trees was made with the program CONSENSUS, which constructed a majority rule tree. This program produces a consensus tree that consists of all groups that occur most often in the replicates. If 95% of trees possessed a given branch point, that branch point was considered significant. For trees constructed from amino acid or nucleic acid sequences of the same genomic region, the tree with the higher confidence level is presented.

RESULTS

New Strains in the Sapporo-like Genogroup

Newly characterized strains in the Sapporo-like genogroup are reported here (Table I), including HuCV-27, HuCV-Lon, and HuCV-Van. The RNA-dependent RNA-polymerase region of these strains was characterized by RT-PCR amplification utilizing primers NV35 and Sapp36, or Sapp35 and Sapp36, followed by cloning and sequencing. Sequence analysis showed that these strains contained the GLPS and YGDD consensus amino acid motifs characteristic of this genomic region (underlined in Fig. 2). Multiple alignment of these new strains with existing Sapporo-like strains in the 3D region revealed an overall nucleotide identity of 55%. The predicted amino acid sequences shared 102 (69%) of the 148 amino acids. Three subgroups appeared to be present among these strains (Fig. 3). The first group included HuCV-Sap, HuCV-DCC, and HuCV-Man, which shared 89–93% nucleotide and 99–100% amino acid identity; the second group included HuCV-Lon and HuCV-Van, which shared 86% nucleotide and 96% amino acid identity; and the third group included HuCV-27, which was isolated from a Houston day care outbreak in 1990.

RT-PCR

Application of NV35 and Sapp36 primers in RT-PCR to nucleic acid extracted from sample HuCV-27 resulted in a double band of products separated by electrophoresis (data not shown), at ~500bp in size. The sequence of the longer PCR product contained the Sapporo-specific analogue of NV35 (Fig. 2). NV35 apparently bound to two sites on this Sapporo-like strain's RNA, 38 nt apart. Sapp35 was subsequently used on other Sapporo-like strains in the RT-PCR tests. This new primer allowed us to characterize the HuCV-Lon strain that did not yield a RT-PCR product using NV35 (Fig. 2). Using Sapp35 and Sapp36, the RT-PCR prod-

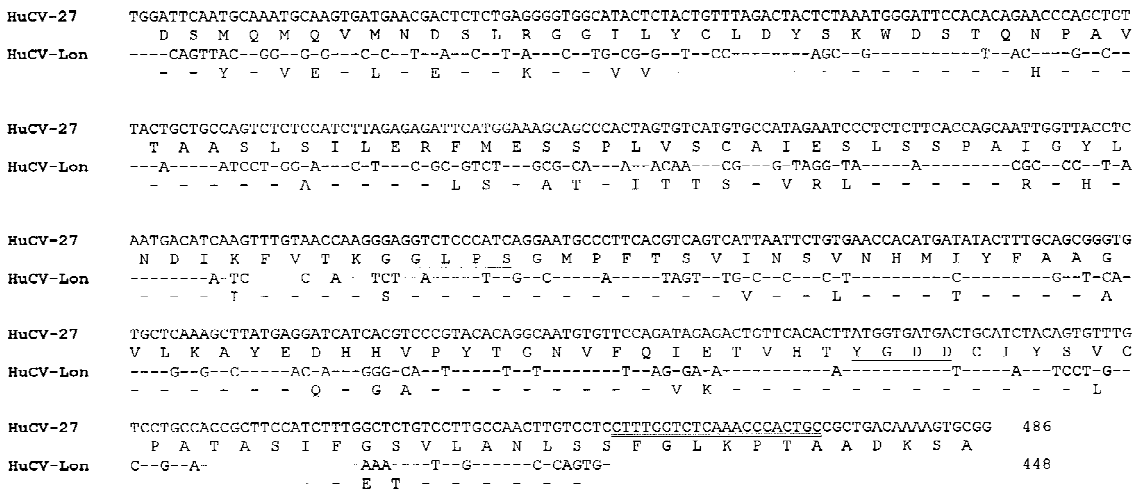

Fig. 2. 3D region nucleic acid and amino acid sequences of a Houston day care center isolate (HuCV-27) and the UK isolate (HuCV-Lon) are shown. A single underline indicates the amino acid motifs (GLPS and YGDD) characteristic of the 3D region; the location of Sapporo primer 35 is double-underlined.

aa% / *nt*%	HuCV-Sap	HuCV-DCC	HuCV-Man	HuCV-27	HuCV-Lon	HuCV-Van
HuCV-Sap	-	93	89	72	68	69
HuCV-DCC	100	-	90	73	68	69
HuCV-Man	99	99	-	73	69	69
HuCV-27	82	82	82	-	67	68
HuCV-Lon	76	76	75	74	-	86
HuCV-Van	75	75	74	71	96	-

Fig. 3. Matrix of pairwise percent nucleotide and amino acid similarity scores of 3D RT-PCR products from the Sapporo-like HuCV strains. Shading is included to highlight more closely related strains.

ucts of Sapporo-like HuCVs were 448bp in size, excluding the primer sequences.

Phylogenetic Analysis of 3D Region

A network of 3D region-based phylogenetic relationships was produced by parsimony algorithms on nucleic acid sequences (Fig. 4). Two large clusters were apparent. One cluster included the SRSVs that contained HuCV-NV, HuCV-KY, HuCV-Sou, HuCV-DS, HuCV-SMA, HuCV-MX, HuCV-Haw, and HuCV-Bri viruses. The other contained the Sapporo-like HuCVs and all the AnCVs except the rabbit hemorrhagic disease virus. HEV-Burma virus and the rabbit hemorrhagic disease virus did not segregate from each other. Some resolution within clusters was apparent. Two subclusters appeared among the AnCVs. One subcluster included only the feline strains, and the other contained the San Miguel sea lion strains together with the primate CV Pan-1. The Sapporo cluster consisted of three subclusters as described in Figure 3.

Phylogenetic Analysis of Capsid Region

The parsimony method applied on the bootstrapped data from the 60/61 capsid region (Fig. 1) sequences resulted in a statistically confident branching pattern. A phylogenetic reconstruction from the same boot-

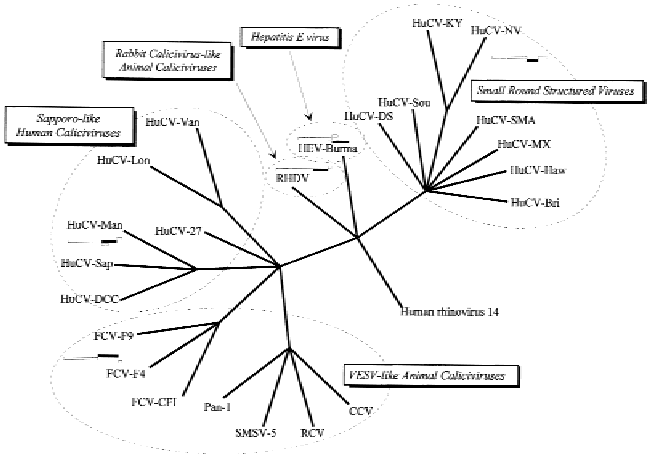

Fig. 4. Parsimony tree constructed for the conserved 3D region. The comparable region of one picornavirus (human rhinovirus) is included as an outgroup. Branches with bootstraps >95% are shown. The lengths of the branches do not represent genetic distances. The distance tree was statistically less confident than the parsimony tree. Also, analyses of amino acid sequences resulted in lower statistical confidence than analyses of nucleic acid sequences. Circles were applied to indicate groups of strains sharing similar genome organization.

strapped data using the Fitch-Margoliash algorithm provided a phylogenetic network representing genetic distances (Fig. 5). Five primary branch points were apparent in the network, indicating three HuCV genogroups and two AnCV genogroups. Within the SRSVs, two subgenogroups were evident. The HEV-Burma virus separated from the rest of the CVs. Two AnCV clusters were found that are named for their prototype strain as the vesicular exanthema of swine virus (VESV)-like and the rabbit CV-like genogroups. However, within the VESV-like genogroup further branching occurred, separating the feline CVs from the SMSVs and Pan-1 [Neill et al., 1991, 1995]. The third HuCV genogroup included the Sapporo-like HuCV

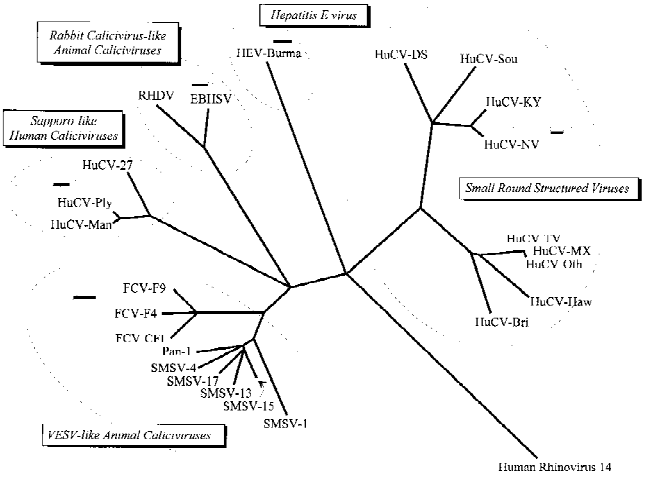

Fig. 5. Fitch-Margoliash distance tree constructed from pairwise distance estimates for the hypervariable capsid region. Percent divergence is represented by the total linear distance between terminal tips. Branches with bootstraps >95% are shown. The comparable region of one picornavirus (human rhinovirus 14) is included as an outgroup. Circles were applied to indicate groups of strains sharing similar genome organization.

strains, which were distinct from other HuCV genogroups and closer to the AnCVs than other HuCVs. The five branch points in the network derived from analysis of nucleotide sequences corresponded to the five different genome organizations observed among CVs [Liu et al., 1995].

DISCUSSION

We initiated phylogenetic analyses of CV genome sequences to establish the position of the Sapporo-like HuCV strains among CVs. Analysis of 3D region sequence identity had placed these strains phylogenetically closer to AnCVs than to other HuCVs [Matson et al., 1995]. However, those analyses and others like them in the literature [Estes and Hardy, 1995; Liu et al., 1995; Matson et al., 1995; Neill et al., 1995; Wang et al., 1994] lacked statistical validity according to accepted methodology for this type of comparison [Felsenstein, 1993]. In order to increase the significance of this analysis, we sought to characterize additional Sapporo-like HuCV strains from Houston, TX, the United Kingdom, and South Africa. Sequences from the RNA-dependent RNA-polymerase region of these strains were incorporated into the phylogenetic analyses. The phylogenetic analyses confirmed the phylogenetic distinctiveness of Sapporo-like HuCVs.

The Sapporo-like HuCVs also were of interest because their genome organization provides a bridge between RHDV and HEV-Burma virus in the number and location of open reading frames [Liu et al., 1995; Jiang et al., submitted]. HuCV Sapporo genomes have four predicted genes: a gene that is a nonstructural polyprotein, a capsid gene fused to the polyprotein, a third gene overlapping the 5′ end of the capsid gene, and a fourth small 3′ end open reading frame. RHDV has genes in three of these four positions, with the nonstructural polyprotein fused to the capsid gene. In hepatitis E virus, the capsid gene is in a distinct frame, a gene overlapping the 5′ end of the capsid gene is present, and the small 3′ end gene is absent.

Assortment of genomes by genome organization and by generation of a phylogenetic tree based upon algorithms that assess sequence identity in a large hypervariable region of the capsid gene yielded concordant conclusions. One significant conclusion of these phylogenetic analyses is a reassessment of the status of hepatitis E virus as a CV. Physical properties and the position of the capsid gene downstream of the nonstructural region led to classification of hepatitis E as a CV [Cubitt et al., 1995]. However, codon usage and the apparently unique genome organization raise concerns about the validity of the assignment [Koonin et al., 1992]. The predicted genome organization of the Sapporo-like HuCVs and the observed distances between CV genogroups in the phylogenetic analyses establish a diversity for CVs that permits inclusion of hepatitis E virus.

In contrast, codon usage in hepatitis E remains distinct. The relationship between some CV genogroups and picornaviruses may provide a clue to the origin of hepatitis E virus. Neill and co-workers [1991] and others [Dinulos and Matson, 1994; Jiang et al., 1993] previously observed statistically significant nucleotide identity, common amino acid motifs, and some common features of genome organization between picornaviruses and feline CVs and SRSVs. However, in picornaviruses the capsid polyprotein gene lies 5′ to the 3′ nonstructural gene, the reverse of the CVs. These shared features suggested the possibility that picornaviruses and CVs arose from a common evolutionary parent by a recombination event. Hepatitis E may be an example of convergent evolution because hepatitis E virus codon usage is so distinct from that of other CV genogroups. It seems reasonable to speculate that hepatitis E virus may be related by recombination to another, as-yet-uncharacterized virus family.

The diversity of the genus *Calicivirus* is such that one must question at what taxonomic level to assign the observed genogroups. Further phylogenetic comparisons suggest that the five genogroups observed here are at least as distinct from each other as different genera in the *Picornaviridae* [Berke and Matson, unpub.], where genome organization is homogenous among genera. How strongly the observed genetic distances and the distinctive genomic organizations "isolate" the strains is unknown, but such isolating mechanisms could be tested by the production of chimeric genomes.

Our results employing phylogenetic algorithms have greater statistical power than studies dependent solely on CLUSTAL [Higgins et al., 1992] previously undertaken to sort CVs [Liu et al., 1995; Neill et al., 1995; Wang et al., 1994]. We studied two regions of CV genomes: the 3D region, for which many strains have been characterized, and the most hypervariable region

of the capsid gene. The 3D region is conserved among CVs, which facilitates RT-PCR amplification of this region, whereas the hypervariable capsid region is much harder to amplify. We conclude that analyses of CVs in the 3D region lack statistical power to sort CVs and caution against conclusions about phylogenetic relationships based upon sequences from this region, especially when the characterized sequences are much smaller than those we analyzed [Ando et al., 1994]. The conclusions from analysis of the 2C genome region made by others [Neill et al., 1995] also need statistical validation. The situation is different in the capsid region analysis where the phylogenetic tree structure was statistically confident and concordant with the assortment of strains by genomic organization.

Our results provide the first comprehensive phylogenetic analyses of the genus *Calicivirus* within the family *Caliciviridae*. CVs appear to include five potential subfamilies. The position of the Sapporo-like HuCVs in the phylogenetic trees indicates that one cannot separate CVs into "human" and "animal" CVs on a phylogenetic basis. The relative positions of the potential subfamilies remain the same whether HEV is included or not. Minor differences in predicted distances occur (data not shown).

It appears unlikely that differences among CVs simply reflect differences in host, target organ, and transmission, i.e., ecologic isolating mechanisms. For example, VESV-like CVs readily move across ocean-land barriers and SRSVs commonly pass through shellfish that apparently can be infected with SMSVs [Smith and Boyt, 1990]. Therefore, additional effort is required to explore the ecology and host range of animal and human CVs, and especially for human health, the possibility that extraintestinal manifestations of SRSV and/or Sapporo-like HuCV infection can occur.

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